Journal of Natural Products
Vol. 58, No. 6, pp. 878-886, June 1995

IDENTIFICATION OF THE GLYCOSIDASE INHIBITORS SWAINSONINE AND CALYSTEGINE B_2 IN WEIR VINE (*IPOMOEA* SP. Q6 [AFF. *CALOBRA*]) AND CORRELATION WITH TOXICITY

RUSSELL J. MOLYNEUX,*

Western Regional Research Center, Agricultural Research Service, USDA, Albany, California 94710

ROSS A. MCKENZIE, BRIAN M. O'SULLIVAN,[1]

Animal Research Institute, Queensland Department of Primary Industries, Yeerongpilly, Queensland 4105, Australia

and ALAN D. ELBEIN

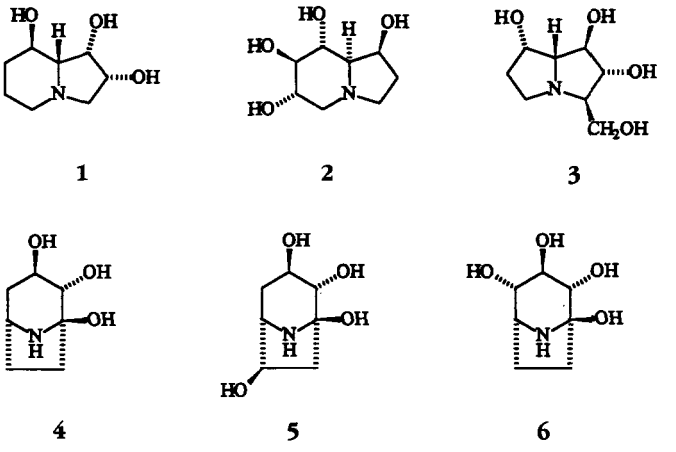
Department of Biochemistry and Molecular Biology, School of Medicine, University of Arkansas, Little Rock, Arkansas 72205

ABSTRACT.—The polyhydroxy alkaloid glycosidase inhibitors swainsonine [**1**] and calystegine B_2 [**6**] have been identified as constituents of the seeds of the Australian plant *Ipomoea* sp. Q6 [aff. *calobra*] (Weir vine) by gas chromatography-mass spectrometry and by their biological activity as inhibitors of specific glycosidases. This plant, which is known only from a small area of southern Queensland, has been reported to produce a neurological disorder when consumed by livestock. The extract of the seeds showed inhibition of α-mannosidase, β-glucosidase, and α-galactosidase, consistent with the presence of **1** and alkaloids of the calystegine class. Histological examination of brain tissue from field cases of sheep and cattle poisoned by Weir vine showed lesions similar to those observed in animals poisoned by the swainsonine-containing poison peas (*Swainsona* spp.) of Australia and locoweeds (*Astragalus* and *Oxytropis* spp.) of North America. These results indicate that Weir vine poisoning is an additional manifestation of the induced lysosomal storage disease, mannosidosis, possibly exacerbated by inhibition of the enzymes β-glucosidase and α-galactosidase by calystegine B_2. This is the first reported example of a single plant species capable of producing structurally distinct glycosidase inhibitors, namely, alkaloids of the indolizidine and nortropane classes.

A number of economically serious livestock poisoning conditions in both the United States and Australia have recently been established as manifestations of induced lysosomal storage diseases, caused by the presence of polyhydroxy alkaloid glycosidase inhibitors in the incriminated toxic plants (1). Predominant among these is the poisoning of cattle, sheep, horses, and other animals by the locoweeds (*Astragalus* and *Oxytropis* spp.) of North America, South America, and China (2), and the poison peas (*Swainsona* spp.) of Australia (3). All of these members of the plant family Fabaceae (Leguminosae) contain the trihydroxy indolizidine alkaloid swainsonine [**1**] (4,5), a potent reversible inhibitor of lysosomal α-mannosidase, ingestion of which produces a phenocopy of the genetic disease, mannosidosis. The structurally related tetrahydroxy indolizidine, castanospermine [**2**], has been isolated from Moreton Bay chestnut or black bean (*Castanospermum australe*) (6), an Australian legume whose seeds are poisonous to livestock and humans. Castanospermine is an excellent inhibitor of α- and β-glucosidase (7), and is accompanied in the plant by several less effective epimers, together with the equally potent α-glucosidase inhibitor australine [**3**], an isomeric polyhydroxy pyrrolizidine (8,9). Although the association of these alkaloids with the toxicity of the plant towards cattle has yet to be demonstrated unequivocally (10), the changes produced are consistent with signs observed in laboratory animals treated with **2** (11).

A novel group of polyhydroxy alkaloids, structurally quite distinct from the

[1]Present address: 5 Karingal Street, Kenmore, Queensland 4069, Australia.



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indolizidine class, has recently been isolated from the bindweeds *Calystegia sepium* and *Convolvulus arvensis* (Convolvulaceae) (12–14). These alkaloids, calystegines A_3 [**4**], B_1 [**5**], and B_2 [**6**], are trihydroxy or tetrahydroxy nortropanes and have been shown to be potent inhibitors of β-glucosidase and α-galactosidase (14). The calystegines have also been reported to occur in a number of *Solanum* species, including potatoes and eggplant, and the leaves and fruits of *Solanum dimidiatum* (potato weed), a toxic plant responsible for a neurological disease of cattle known as "Crazy Cow Syndrome" in west Texas (15–17). While the bindweeds have not been associated with serious toxicity problems, a recent report has implicated *C. arvensis* in weight loss and intestinal fibrosis in horses in Colorado (18). However, the only alkaloids detected in the aerial parts of the plant were the simple tropane alkaloids tropine, pseudotropine, tropinone, cuscohygrine, and hygrine, rather than the calystegines, and no glycosidase inhibitory activity was detected in the extract. In contrast, literature reports of toxicity in cattle and sheep caused by Weir vine (*Ipomoea* sp. Q6 [aff. *calobra*]) in Australia were indicative of a lysosomal storage disease. Weir vine was first reported to be toxic to animals by White (19), who described reports from local graziers of nervous derangement in sheep, cattle, and horses that had eaten the plants. Toxicity was confirmed in feeding experiments with sheep, in which consumption of about 7 kg wet wt of plant daily for five weeks resulted in clinical signs consistent with natural cases (20). Necropsy of these animals revealed nephrosis, but no examination of the nervous system was reported. The inclusion of the genus *Ipomoea* in the family Convolvulaceae suggested to us that the calystegines might be the responsible constituents. The availability of a small collection of Weir vine seeds from the Maranoa pastoral district of southern Queensland provided an opportunity to examine the total alkaloid extract in detail, in order to determine whether or not such polyhydroxy nortropane alkaloids might be present.

RESULTS AND DISCUSSION

Extraction of the ground seeds of Weir vine with MeOH, followed by ion-exchange chromatography on Dowex 50W-X8, gave the total alkaloid fraction as a yellow oil. In earlier work forcing reaction conditions were used to achieve per-trimethylsilylation of the calystegines for gc-ms analysis (14). Due to subsequent difficulty experienced in achieving complete derivatization, milder conditions and shorter reaction times were used which left the secondary amino group unsubstituted. Analysis of the trimethylsilylated alkaloid extract thus prepared by capillary gc-ms, showed the presence of two major components with retention times of 14.21 (23%) and 16.25 (31%)

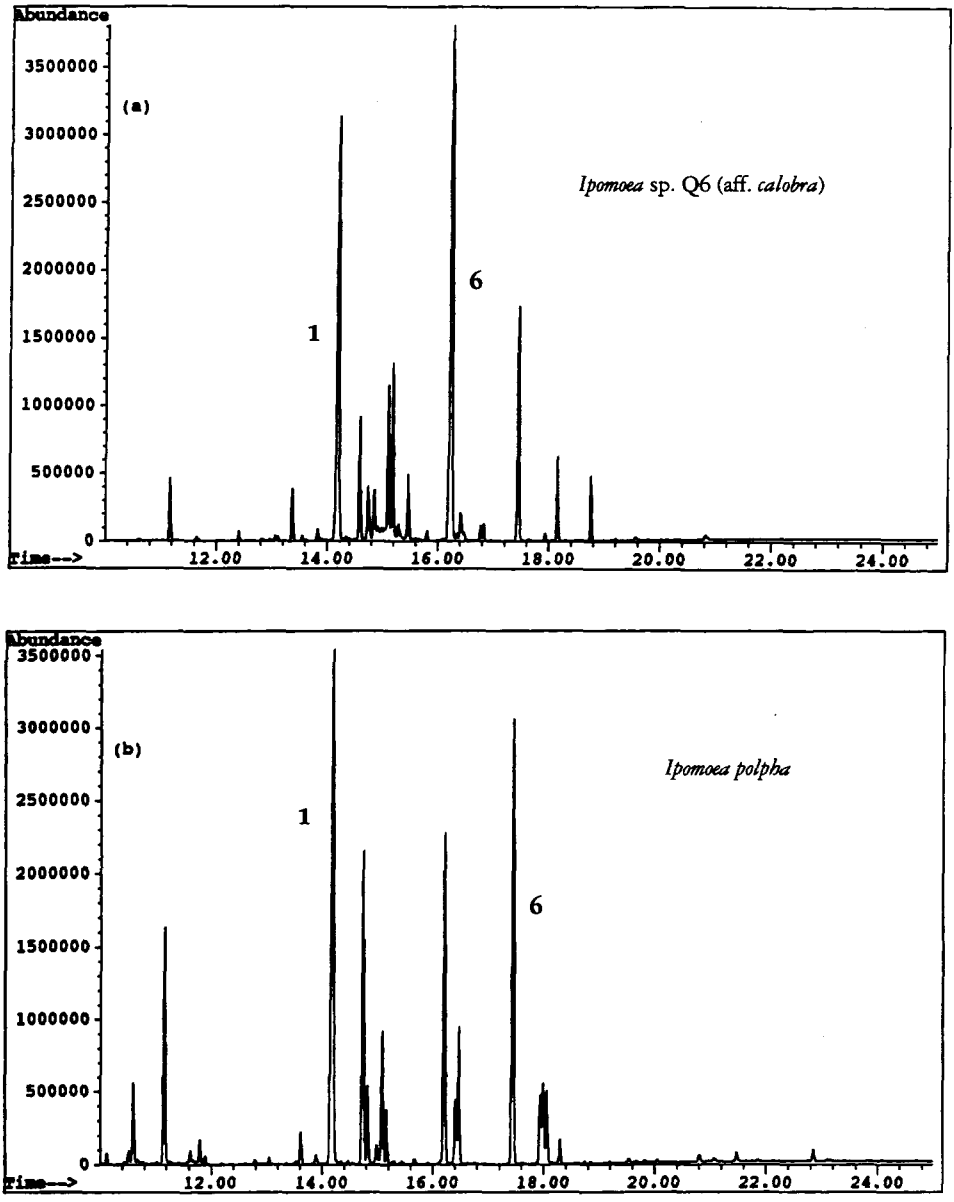

FIGURE 1. Analysis of trimethylsilylated alkaloid extracts by gc-ms. (a) Total ion chromatogram of Weir vine (*Ipomoea* sp. Q6 [aff. *calobra*]) seed; (b) Total ion chromatogram of *Ipomoea polpha* seed.

min, together with a less significant component at 17.46 (9%) min (Figure 1a). The later-eluting major peak had a retention time and mass spectrum (Figure 2a) identical in all respects to that of the tetra-TMSi derivative of a standard sample of calystegine B_2. This derivative exhibited a mass spectrometric fragmentation pattern which was quite different from that previously reported for the penta-TMSi derivatives (14). Whereas the latter showed a base peak at *m/z* 229, the partially silylated derivative had a base peak at *m/z* 217. This fragment is commonly observed in carbohydrate and inositol TMSi derivatives, in which three adjacent trimethylsilylated hydroxyl groups give rise to the ion $TMSiOCH{=}CH\text{-}CH{=}{}^{+}OTMSi$ (21,22), and is thus consistent with the structure of calystegine B_2 [**6**]. The minor component with R_t 17.46 min had a similar

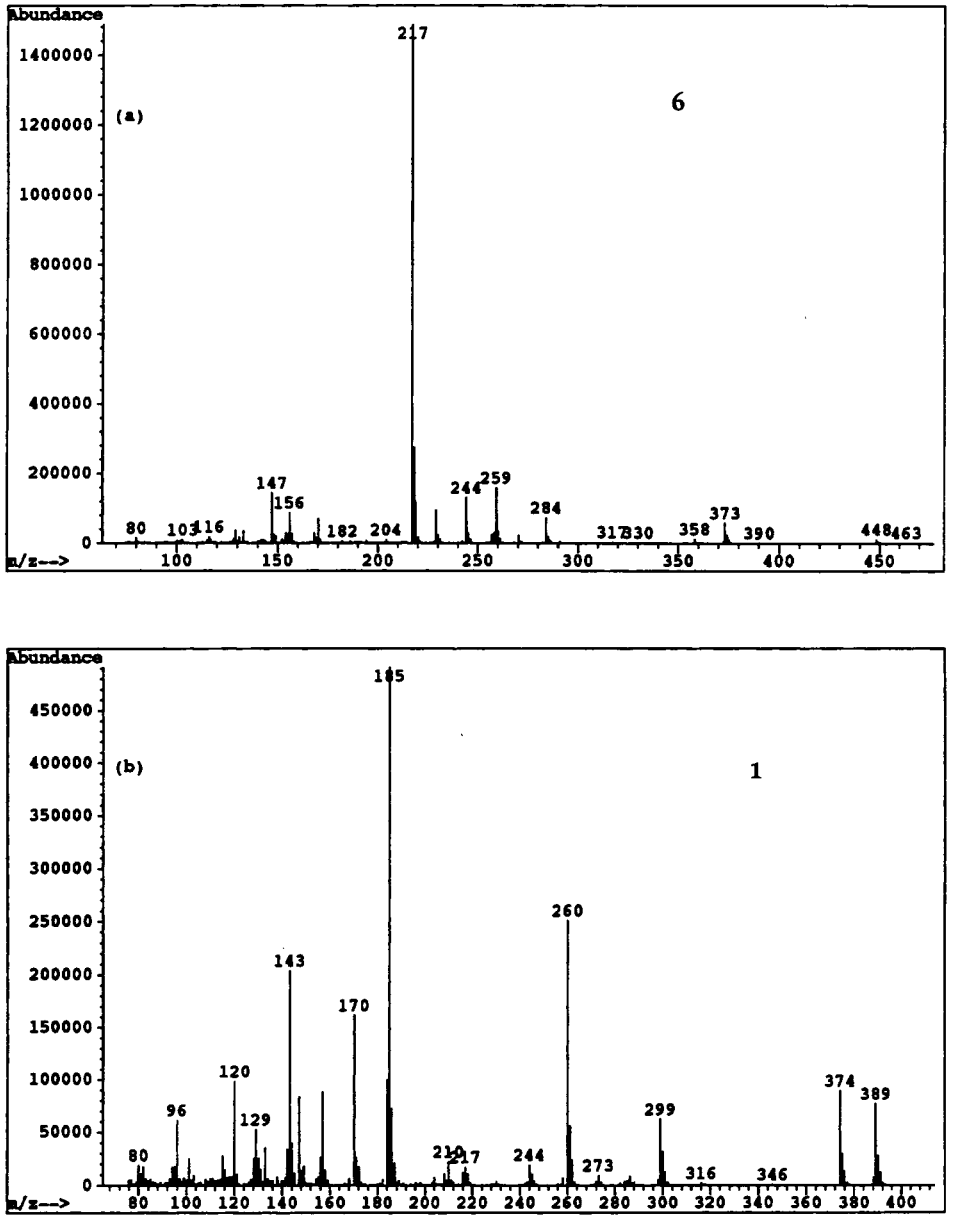

FIGURE 2. Mass spectra of components of Weir vine. (a) Peak at 16.25 minutes (tetra-TMSi calystegine B_2); (b) Peak at 14.21 minutes (tri-TMSi swainsonine).

fragmentation pattern to that of calystegine B_2 but showed a molecular ion at *m/z* 551, indicating the presence of an additional trimethylsilylated hydroxyl group. The alkaloid is therefore a pentahydroxy calystegine, possibly identical to calystegine C_1, which has recently been isolated from the leaves of *Morus alba* (23).

The earlier eluting (14.21 min) major peak did not correspond in retention time to any of the TMSi derivatives of available calystegine standards, nor did the mass spectrum show fragments characteristic of this class of compounds (Figure 2b). Thus, under the same chromatographic conditions tri-TMSi calystegine A_3 and tetra-TMSi calystegine B_1 had retention times of 13.43 and 15.12 min, and showed molecular ions at *m/z* 375

and 463, respectively. The molecular ion at *m/z* 389 and the base peak at *m/z* 185 were inconsistent with both a nortropane structure and any underivatized hydroxyl or amino groups. It was with considerable surprise that we subsequently recognized the fragmentation pattern as being the same as that of the tri-TMSi derivative of **1** (24), and analysis of a pure standard of this alkaloid gave an identical mass spectrum and retention time of 14.21 min. The identity of this component of the Weir vine extract with **1** was further confirmed by analytical tlc on Si gel, under which conditions both the alkaloid extract and a pure standard had an R_f value of 0.44 and gave a typical purple spot on spraying the plate with Ac_2O/Ehrlich's reagent (2).

In order to obtain a measure of the swainsonine content of the seeds, the extract was analyzed by gc with flame ionization detection, for which a response curve had been developed with an external standard of pure **1** (24). The content of **1** was thereby established as 0.048% of the dry wt of the plant material. This value is quite comparable with the level determined for a number of locoweed species well-established as toxic to livestock. Moreover, it has been estimated that locoweeds containing at least 0.001% swainsonine are capable of producing neurological damage if consumed over a sufficient period of time (16) and the content in Weir vine therefore far exceeds this level. Insufficient pure calystegine B_2 was available for a gc response curve to be determined for this alkaloid, but by assuming the detector response to be the same as that of **1**, the level in the seeds was estimated as 0.058%.

Although the analytical evidence established the presence of both swainsonine and calystegine B_2 in Weir vine seeds, insufficient plant material was available to isolate each alkaloid and establish that their absolute stereochemistries were identical with the alkaloids first isolated from *Swainsona canescens* and *Calystegia sepium*, namely (−)-swainsonine and (+)-calystegine B_2 (4,13). In an attempt to resolve this question, the inhibitory properties of the alkaloidal extract of the seeds were therefore evaluated against a series of hydrolytic enzymes. The extract proved to be a good inhibitor of α-mannosidase, β-glucosidase, and α-galactosidase but showed no significant inhibition of amyloglucosidase, α-glucosidase, β-galactosidase, or β-mannosidase (Table 1). These results are entirely consistent with the analytical evidence and provide presumptive evidence that the alkaloids present in Weir vine are indeed (−)-swainsonine [**1**] and (+)-calystegine B_2 [**6**], since it is unlikely that their enantiomers would exhibit similar inhibitory properties. Based upon the gc analytical results the activity of **1** in the extract

TABLE 1. Inhibition of Glycosidases by Weir Vine Extract.

Amount of Extract (μg)	Activity of Enzyme (OD at 410 nm)						
	α-Glc	Amylo-Glc	β-Glc	α-Gal	β-Gal	α-Man	β-Man
None	3.07	—	2.45	1.29	—	2.13	—
0.1	2.62	—	2.75	0.92	—	1.41	—
0.2	2.62	—	2.60	1.04	—	1.07	—
0.3	3.21	—	2.10	0.94	—	0.92	—
None	3.29	—	2.92	1.06	—	2.07	—
0.5	2.86	—	1.75	0.84	—	0.62	—
1.0	2.61	—	1.35	0.77	—	0.36	—
2.0	2.90	—	1.10	0.63	—	0.23	—
3.0	2.90	—	0.75	0.53	—	0.17	—
None	3.27	1.84	2.52	1.53	1.84	2.08	2.56
5.0	1.99	1.60	0.62	0.44	1.51	0.13	2.56
10.0	1.05	1.53	0.32	0.34	1.44	0.13	2.48
20.0	0.45	1.38	0.24	0.27	1.25	0.11	2.31
30.0	0.21	1.23	0.16	0.21	0.93	0.11	2.58

to produce 50% inhibition of α-mannosidase was calculated to be 40 ng/ml, while pure **1** showed corresponding activity at 25–30 ng/ml (25). Similarly, the calystegine B_2 in the extract was estimated to be active towards β-glucosidase and α-galactosidase at 0.5 and 1.4 μg/ml, respectively, whereas the pure alkaloid showed 50% inhibition at 0.6 and 1.2 μg/ml (14).

The analytical evidence supported our hypothesis that the toxicity of Weir vine is a consequence of the presence in the plant of glycosidase-inhibitory enzymes. Additional confirmatory evidence was provided by a retrospective histological examination of brain tissue preserved from field poisoning cases in 1973 and 1975 of one sheep and two cattle. The tissues revealed swollen axons and fine cytoplasmic vacuolation of neurons similar to lesions seen in animals poisoned by plants that contain swainsonine, such as *Swainsona, Astragalus,* and *Oxytropis* (26). These results indicate that Weir vine poisoning is an additional manifestation of the induced lysosomal storage disease, mannosidosis, possibly exacerbated by inhibition of β-glucosidase and α-galactosidase by calystegine B_2. Inhibition of the latter enzymes would produce phenocopies of the genetic lysosomal storage defects Gaucher's disease and Fabry's disease, respectively. Significant indicators of these diseases are epileptiform seizures and vacuolation of Purkinje cells. Although muscle-twitching, trembling, and "star-gazing" have been reported in field cases of the poisoning (27), confirmation of this hypothesis must await controlled feeding experiments and comprehensive histological and biochemical examination of selected tissues.

The botanical classification of Weir vine is problematic. It is known only from a small area of the Maranoa pastoral district of southern Queensland, approximately centered on 27°30′S, 148°30′E. Further study could classify this taxon as a subspecies of *I. polpha*, which has similarly restricted populations in north-eastern Queensland and possibly in the Northern Territory of Australia (28). These three populations of *Ipomoea* are practically indistinguishable using morphology, and all grow in similar habitats and soil types (R.W. Johnson, 1994, personal communication). The co-occurrence of two glycosidase inhibitory alkaloids of such distinctly different structural types in a single plant species such as Weir vine is exceptional. While a precedent appears to exist in the co-occurrence of castanospermine [**2**] and australine [**3**] in *Castanospermum australe*, there is an obvious biosynthetic relationship between these two isomeric alkaloids. No such relationship can by hypothesized for **1** and **6**, which differ not only in the mode of ring fusion but also in the number of hydroxyl groups present. The presence of these two alkaloids, belonging to the indolizidine and nortropane classes, respectively, may therefore provide a specific chemotaxonomic marker for more complete botanical identification of these species. Unfortunately, a severe drought in Queensland has rendered collection of plant material from multiple sites for such a study impractical at the present time.

However, a small sample of seed from *I. polpha,* collected in 1979 in the Northern Territory, was obtained and analysis by gc-ms of the alkaloid extract isolated from these seeds, under the same conditions as used for the Weir vine collection, showed the presence of four major components with retention times of 14.20 (27%), 14.74 (11%), 16.22 (12%), and 17.46 (17%) min (Figure 1b). The signals at 14.20 and 16.22 min corresponded in retention time and mass spectra to the TMSi derivatives of swainsonine and calystegine B_2. The signal at 14.74 min showed a mass spectrum very similar to that of calystegine B_2, suggesting that it is an isomer thereof, but not calystegine B_1, which has an R_t of 15.12 min. The compound eluting at 17.46 min had a retention time and mass spectrum identical to that of the minor alkaloid detected in the Weir vine extract and may therefore also be a pentahydroxylated calystegine C. Quantitative analysis of the alkaloids in *I. polpha* by capillary gc with fid indicated swainsonine and calystegine B_2

contents of 0.107% and 0.025% respectively, and the swainsonine level is therefore twice that in Weir vine, whereas the calystegine B_2 level is only half as much. This evidence suggests that *I. polpha* should be at least as toxic as Weir vine, if grazed.

The co-occurrence of **1** and **6** in both seed samples denotes a close chemotaxonomic relationship between *Ipomoea* sp. Q6 [aff. *calobra*] and *I. polpha*, but the differences in the composition of the minor constituents are sufficient to suggest that they are probably not the same species. Collection and analysis of plant material from all populations in question is required in order to fully resolve this taxonomic problem. In addition, the occurrence of these toxic alkaloids in Weir vine necessitates examination of other members of the genus *Ipomoea*, to ensure that they do not present a hazard to humans through those species used as food plants.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The identity of alkaloids was established by gc-ms of the TMSi derivatives, prepared by treatment with MSTFA in pyridine at 60° for 1 h, on a Hewlett-Packard 5890 Series II instrument equipped with a 5971 mass-selective detector operating at 70 eV, on-column injector, and a 60-m×0.32-mm i.d. SE-30 fused silica column. The column was temperature programmed from 120° to 300° at 10°/min. The amount of **1** present in the extract was determined by measuring the response factor for an external standard of the pure alkaloid TMSi derivative on a Hewlett-Packard 5830 instrument with flame-ionization detection, on-column injector, and a 30-m×0.32-mm i.d. SE-30 fused silica column temperature programmed from 120° to 300° at 5°/min (24). The presence of **1** in the extract was confirmed by analytical tlc on 0.25 mm Si gel plates developed with $CHCl_3$-MeOH-NH_4OH-H_2O (70:26:2:2) and detected by spraying with Ac_2O followed by Ehrlich's reagent (2).

PLANT MATERIAL.—Seeds of Weir vine were collected in March 1994 at 27°15′S, 148°03′E, on the western edge of the plant's known distribution in the Maranoa pastoral district. A voucher specimen (No. AQ625698) is deposited in the Queensland Herbarium under the name *Ipomoea* sp. Q6 (aff. *calobra*). The *I. polpha* seeds were collected in January 1979 at S. Tin Fish Well, Stirling Station, Northern Territory, and can be related to a Queensland Herbarium voucher specimen through the collector's number (P.K. Latz 8252).

EXTRACTION AND ISOLATION.—Mature Weir vine seeds (1.055 g) were ground to pass a 1-mm screen, extracted with MeOH for 16 h in a Soxhlet apparatus, and the alkaloidal fraction was purified by ion-exchange chromatography on a 5-×0.5-cm column of Dowex 50W-X8 (NH_4^+ form) (9). The 0.5% aqueous NH_4OH eluate was evaporated to dryness, redissolved in distilled H_2O, and made up to a volume of 10 ml in a volumetric flask. Aliquots of this solution were evaporated to dryness and derivatized by MSTFA. Analysis by gc-ms of the TMSi derivative thus obtained showed two primary components, with R_t 14.21 (23%) and 16.25 (31%) min, a secondary component (9%) at 17.46 min, and several minor constituents (Figure 1a). The relative composition was determined as percent area of the total chromatogram. Two additional extractions were performed on the same scale, without quantitation, in order to ensure that the detection of **1** was consistent and not due to inadvertent contamination. Both **1** and **6** were identified unequivocally in each experiment. Mature *I. polpha* seeds (1.027 g) were extracted, derivatized, and analyzed in a similar manner. The gc-ms showed four primary components, with R_t 14.20 (27%), 14.74 (11%), 16.22 (12%), and 17.46 (17%) min, and a number of minor constituents (Figure 1b).

Characterization of calystegine B_2 [**6**].—The peak with R_t 16.25 min on gc-ms had a retention time identical to that of the TMSi derivative of **6**. Co-injection with an authentic sample gave a single peak with the same retention time. TMSi derivatives of calystegine A_3 and calystegine B_1, prepared under the same conditions, gave peaks with R_t 13.43 and 15.12 min, respectively. Tetra-TMSi calystegine B_2, eims *m/z* $[M]^+$ 463 (0.1), 448 (1), 373 (4), 284 (5), 259 (11), 244 (9), 229 (6), 217 (100), 156 (6), and 147 (10) (Figure 2a).

Characterization of swainsonine [**1**].—The peak with R_t 14.21 min on gc-ms had a retention time identical to that of the TMSi derivative of **1**. Co-injection with an authentic sample gave a single peak with the same retention time. Tri-TMSi swainsonine, eims *m/z* $[M]^+$ 389 (16), 374 (18), 299 (13), 260 (51), 185 (100), 170 (33), 157 (18), 147 (17), and 143 (41) (Figure 2b) (24). Analytical tlc of a sample of the alkaloid extract of the seeds and an authentic sample of **1** gave purple spots characteristic of polyhydroxy indolizidine alkaloids, on spraying the plate with Ac_2O/Ehrlich's reagent (2), and identical R_f values of 0.44.

QUANTITATION OF **1** AND **6**.—Pure **1**, analyzed as the TMSi derivative by gc-fid, gave a linear response over the range 25–200 ng, with an average response of 876 counts/ng. A portion (100 μl) of the standard

extract from the Weir vine seeds was evaporated to dryness under a stream of dry N_2 and derivatized in pyridine (100 μl) and MSTFA (100 μl). Three 2.0-μl aliquots of the derivatization product were analyzed by gc-fid, giving an average of 44,297 counts for **1** and 53,350 counts for **6**. These values are equivalent to 50.6 and 60.9 ng, respectively, in the sample injected and extrapolate to 0.506 mg (0.048%) **1** and 0.609 mg (0.058%) **6** in the sample of seed originally extracted.

The *I. polpha* seeds were analyzed in an identical manner giving an average of 95,920 counts for **1** and 22,650 counts for **6**, corresponding to 1.095 mg (0.107%) **1** and 0.258 mg (0.025%) **6** in the sample of seed originally extracted. The levels of the incompletely characterized alkaloids with R_t 14.74 and 17.46 min were calculated to be 0.029% and 0.031%, respectively.

GLUCOSIDASE INHIBITION.—Amyloglucosidase (from *Aspergillus niger*), α-glucosidase (from yeast), β-glucosidase (from almonds), α-galactosidase (from *A. niger*), β-galactosidase (from bovine liver), α-mannosidase (from jack bean), and all *p*-nitrophenyl glycoside substrates were purchased from either Boehringer Mannheim Biochemicals or the Sigma Chemical Company. β-Mannosidase was purified from *A. niger* as previously described (29). The enzymatic activities of the arylglycosidases were determined colorimetrically by monitoring the release of *p*-nitrophenol from the appropriate *p*-nitrophenyl glycoside substrate (30). A portion (2.0 ml) of the standard extract from the seeds was evaporated to dryness under a stream of dry N_2, yielding 0.5 mg of residue. The inhibitory activity of this material against the above enzymes was determined by a series of serial dilutions (Table 1). The amount of material required to produce 50% inhibition of α-mannosidase, β-glucosidase, and α-galactosidase was found to be 0.2 μg, 1.0 μg, and 3.0 μg, respectively. All other enzymes tested showed 50% inhibition only at levels of 10 μg or above.

ACKNOWLEDGMENTS

The collection of seeds and a voucher specimen of *Ipomoea* sp. Q6 [aff. *calobra*] by D.W. Burton, Department of Primary Industries, Mitchell, Queensland, is gratefully acknowledged. The *I. polpha* seeds were kindly provided by Dr. R.J. Johnson. We thank Prof. F.R. Stermitz for informing us of the results of research on the toxicity of *Convolvulus arvensis* in horses prior to publication. This research was supported in part by a grant from the National Institutes of Health (HL-17783).

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Received 11 January 1995